# The Electron-Micrographic Structure of Shadow-Cast Films and Surfaces

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The lower limit of size of biological objects which can be photographed with the electron microscope, by the aid of shadow-casting, is shown to depend upon the smoothness of substrate upon which they can be mounted, and upon the continuity of structure of the thin films with which they are shadowed. Numerous attempts to improve the existing deficiencies are reported, both with respect to producing smoother substrate films, and to producing films for shadow-casting of high efficiency and continuity of structure.

No success has been encountered in producing a usable substrate film perceptibly smoother than the collodion and Formvar films commonly used. It is found that the best shadow-casting material for this type of film is uranium or uranium oxide.

Verification has been obtained of the severe granulation of gold films previously used in the pre-shadowed replica process, when subjected to the electron current of a biased-beam electron gun. Attempts to reduce the granulation to a satisfactory level have failed. Various methods of preparing pre-shadowed replicas are reported. The factors affecting the tenacity of evaporated films to glass surfaces are discussed. It is found that elements which oxidize readily are relatively adherent to glass, while the elements gold, palladium, platinum, and rhodium are not. Uranium sulfide can be used as a pre-shadowed replica material, but only with some uncertainty, owing to its chemical instability. It has been found that a palladium-platinum mixture is the most satisfactory material for use in the pre-shadowed replica technique, and that films of this mixture in a thickness of about 6A produce adequate shadows in which there is no sign of granulation. The surface of clean glass is again found to have the smoothest structure of any material known, with practically no sharp discontinuities in elevation as great as 10A.

An appendix is given, in which technical details of shadow-casting and replica production are described.

## INTRODUCTION

IN 1944 Williams and Wyckoff[1] demonstrated that the electron micrography of minute biological objects could be greatly improved by the application of a technique which has come to be called "shadow-casting" or "shadowing." Very small particles of substances of organic nature, such as are found in biological material, ordinarily do not have sufficient electron scattering power to make their detection possible when they are mounted on a collodion film substrate in the usual manner of specimen preparation. Consequently, prior to the advent of the shadowing technique, the electron microscope could not be utilized to the full extent of its inherent resolving power in the examination of small, particulate biological material, and, in fact, it was generally accepted that a particle diameter of about 200A was the lower limit of detectability. This is in marked contrast to the minimum discernible diameter of particles of a substance of high atomic number, such as a gold sol, where a size on the order of 10A has been reported.[2]

In the shadow-casting process the specimen is coated, in vacuum, by the oblique deposition of a layer of a material relatively opaque to electrons, such as chromium. As a result any particle which is elevated above the general layer of the substrate film prevents the formation of the chromium layer in the immediately adjacent region opposite to the direction of the source of chromium, creating an area which might be termed a metallic "shadow." When the shadowed specimen is photographed in the electron microscope, the areas relatively devoid of the metallic film are more transparent to the electrons than the adjacent areas of the substrate, and hence appear dark on the photographic negative, while the object casting the shadow appears relatively white. The general effect is the same as that obtained in a positive photograph of a region reflecting light under oblique illumination.

Williams and Wyckoff were able to photograph with considerable clarity objects of organic nature having diameters of approximately 100A, by the use of gold as the shadowing agent, and collodion as a substrate film. They found, however, that this technique was not applicable in its simple form to the detection of objects

[1] R. C. Williams and R. W. G. Wyckoff, J. App. Phys. 15, 712 (1944).
[2] James Hillier, J. App. Phys. 17, 307 (1946).

smaller than 100A, owing to the lack of smoothness of the surface of the collodion film, which has a "pebbly" structure of the order of 50A in size. They developed a technique which they called a "pre-shadowed replica" method,[3] in which the shadowing is first done with the specimen material mounted on a glass surface. Subsequently the specimen and shadowing layer are stripped from the glass by the use of a collodion film, and effectively are transferred to the surface of that film. This procedure was very satisfactory at the time, since the surface of polished glass was found to be extremely smooth, and the inherent surface structure of the collodion film was not exhibited because it was not shadowed.

Fairly recently there has been developed for use in the electron microscope a biased-beam electron gun[4] which has a desirable characteristic of producing an intense electron beam in the plane of the specimen. However, the intense electron beam from this type of gun has been reported[5] to produce an agglomeration of the gold film used in shadowing, with the result that the film appears broken into minute globules; an effect commonly called "granulation." The advantages of the biased beam are too great to consider abandoning it in order to preserve the usefulness of gold films, and hence the problem of suitable shadowing agents and specimen substrates has had to be examined anew. In particular, our problem has been to develop methods whereby the presence and approximate shapes of biological particles of molecular weights around 150,000–200,000 (one dimension less than 40A) can be ascertained.

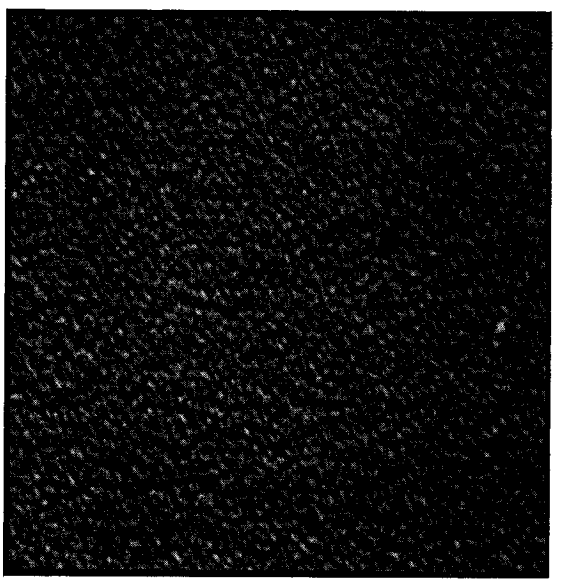

FIG. 1. A water-spread surface of collodian, shadowed with a film of uranium about 6A thick at a grazing angle of 11° (a "5 to 1" angle). Scale mark represents 0.1 micron.

If only a sufficiently smooth substrate film existed, the problem of photographing particles of diameters less than 50A would be solved very simply. Materials exist for shadowing which do not granulate (as will be discussed later), and they are of such high atomic number and density that films only 4–6A thick, as measured normally to the substrate, will cast shadows of sufficient contrast to be usable. On the other hand, the pre-shadowed replica method alone would be a good solution, since the surface of clean glass is adequately smooth, if only there could be found substances for shadowing which did not granulate and could be readily stripped from the glass to form the necessary replicas.

Our attempts to solve the problem have taken two obvious paths: (1) the search for a smooth substrate film to be used in direct shadowing and (2) the search for a proper shadowing material to be used in making pre-shadowed replicas.

## II. ATTEMPTS TO IMPROVE THE SURFACE SMOOTHNESS OF SUBSTRATE FILMS TO BE USED FOR DIRECT SHADOW-CASTING

### A. Shadow-Casting Substances Which Do Not Granulate

Collodion or Formvar (polyvinal formal) films are commonly made for use in electron microscopy by first forming the film on water, and transferring this to the fine-mesh microscope screen. As has been indicated earlier, such films are entirely satisfactory for biological objects of particulate nature, if they have diameters greater than about 100A, and for relatively large bio-

[3] R. C. Williams and R. W. G. Wyckoff, Science **101**, 594 (1945).
[4] James Hillier and R. F. Baker, J. App. Phys. **16**, 469 (1945).
[5] R. J. Mandle, Proc. Soc. Exp. Biol. Med. **64**, 362 (1947).

logical objects whose surface detail is the main point of interest.

If fine detail in the specimen is to be observed, however, three conditions are imposed upon the shadowing agent. It should have a high scattering power for electrons, so as to be usable in a very thin layer; it should be readily evaporated in the vacuum chamber used for shadow-casting; it should not granulate under the impact of the electron beam. As several investigators have shown, the elements of highest melting point are least likely to granulate, and the obvious material to use to satisfy the first and last conditions mentioned above is uranium.[6] Its only objectionable characteristic is the relative difficulty with which it is evaporated, since it alloys readily with the tungsten filament.

We have reconsidered the use of uranium, and find, in agreement with others, that a thin uranium film is completely oxidized upon any exposure to air, and certainly by the time it is examined in the electron microscope. It seems entirely reasonable, then, to suppose that uranium oxide ($U_3O_8$) is a satisfactory material to evaporate in the first place, and we have found it to be so, particularly inasmuch as it does not perceptibly attack the tungsten filament. When deposited in a thickness calculated to be only 6A, it produces faint, but discernible, shadows. Tungstic oxide can also be readily evaporated, and makes a somewhat less satisfactory shadowing agent than uranium oxide, owing to its lower average atomic number. A disadvantage of the use of uranium oxide is its very high vaporizing point, resulting in the possibility of damage to the specimen if it is placed too near the filament during shadow-casting.

### B. Attempts to Reduce the Irregularities of the Substrate Film

Both collodion and Formvar are commonly used as substrate films, but inasmuch as we have never detected any systematic difference in their surface textures, we shall refer mostly to collodion with the understanding that the same conclusions apply to Formvar. The structure of a collodion film (U.S.P., diluted about 10-fold with amyl acetate) spread on water, is shown in

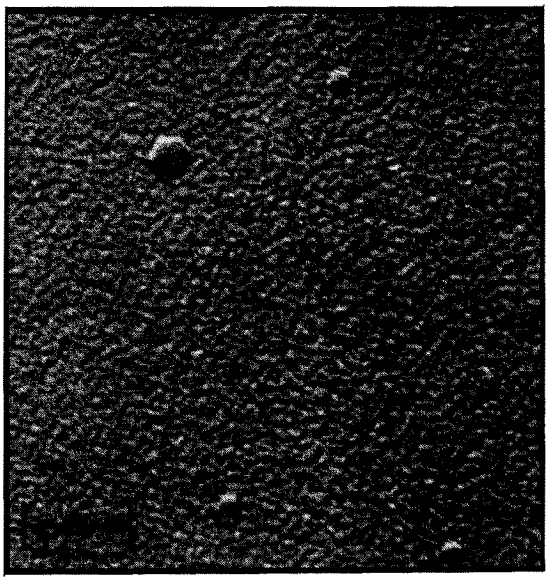

FIG. 2. The characteristically rough surface of a thick film deposited in vacuum. The example shown in this micrograph is that of a collodian replica of a film of aluminum 1000A thick. Replica shadowed with uranium at a 5 to 1 angle. Scale mark represents 1 micron.

Fig. 1. The surface was shadowed with about 6A of uranium, at a 5 to 1 angle. The considerable irregularity of its surface is evident, and it is clear that particulate objects around 50A in diameter that might exist in a preparation would not be detectable when masked by the structure of the collodion background. We have compared the air-exposed surface of spread films of collodion with the water-exposed surface, and find no difference in structure.

a. We have prepared collodion films by allowing them to dry from the solvent while in contact with a smooth solid surface, such as glass. The film can then be stripped off and the side which has dried in contact with the glass subsequently shadowed. This method has been tried with both collodion and Formvar, and with amyl acetate, dioxane, and ethylene dichloride as solvents, but the surface structure always has been found to have irregularities of the same magnitude as shown in Fig. 1. Evidently the highly polymerized units in both types of film retain their identity, form, and interwoven structure following the evaporation of the solvent.

b. Another possible method of reducing the surface structure of the supporting film is to coat

[6] R. C. Williams and R. W. G. Wyckoff, J. App. Phys. 15, 712 (1944).

the collodion film with another film whose inherent surface structure might be expected to be finer, with the hope that the collodion structure would be filled in or bridged over. A promising material for this purpose is a film of silicon monoxide deposited in vacuum. It is known that films of SiO are relatively structureless, as observed by electron diffraction, and that they are sufficiently strong to serve as substrate films when only 20A thick.[7] We have found however, that when films of SiO are deposited upon collodion, in calculated thicknesses varying from 20 to 100A, the structure of the collodion surface is imparted to the SiO layer, and no smoothing of the former is effected.

c. There is no inherent reason why the substrate film must be formed of collodion or Formvar, and some work has been done with films of other materials. Ellis[8] has reported on the surface structure of a low polymer of polyethylene, shadowed with chromium, and has found rather gross irregularities. We have tried polyethylene dissolved in warm xylene and cast as a film on hot water, and also flowed as a film upon hot, smooth glass. In both cases the surface irregularities, as photographed subsequent to shadowing with uranium, have been found to be larger than those of collodion.

d. We have experimented with the use of a film of SiO deposited directly upon glass, in order to ascertain whether or not the glass-deposited surface is sufficiently smooth to be useful. However, a film of SiO cannot readily be stripped from clean glass, and we have tried pre-coating the glass with very thin films of soluble materials such as sodium chloride, boron, and copper to make possible the removal of the SiO film. In every case we have found that, if the sub-film is thick enough to allow the SiO to be removed, it is then thick enough to introduce into the SiO surface the characteristically rough structure of a thick film deposited in vacuum (Fig. 2).

## III. IMPROVEMENT OF PRE-SHADOWED REPLICAS

### A. The Granulation of Gold

We have performed some experiments with gold-shadowed replicas, to assure ourselves of the conditions under which the granulation of the gold occurs. We find that these conditions are: too great a thickness of the gold film, deposition of the film in a poor vacuum, and, most importantly, an electron beam current of too high intensity. It is our experience, in accord with that of others, that *if an electron microscope equipped with a biased-beam gun is to be used for the examination of minute structures, the use of gold as a shadowing agent is inadvisable.*

### B. Experiments on the Possible Reduction of Gold Granulation

Gold is a very convenient shadowing material, easy to evaporate, of high atomic number, and readily stripped from the glass. We have felt that its use should not be abandoned without investigating the possibility that its granulation could be decreased below a troublesome value. Two possible approaches are evident: to attempt to stabilize or fix the gold film before it has been stripped from the glass by the collodion film, and to attempt to stabilize it subsequent to stripping but prior to insertion in the microscope.

Whether or not these procedures appear naïve hinges upon one's conception of what occurs when an originally continuous metallic film aggregates into droplets at temperatures several hundred degrees below its melting point. The phenomenon is apparently one of atomic diffusion, but there is complete ignorance as to what might serve as centers upon which the small aggregates form. An analogous phenomenon is the "sintering" or aggregation of a gold black at a temperature some 800°C below the melting point of bulk gold. It has occurred to us that a substance which would interfere with the free diffusion of the gold atoms, or would inhibit the beginnings of the formation of aggregates, might prevent the customary granulation.

a. In an attempt to stabilize the gold film before stripping, two materials of high melting point, SiO and uranium, were deposited along with the gold. Films of these were applied before, after, and during the deposition of the gold. When they were deposited *before*, and if made thin enough to allow the gold to be stripped off, they did not prevent granulation. *Mixed* with the gold film they appeared to have no effect, at least until made such a high percentage of the

[7] C. E. Hall, J. App. Phys. **19**, 198 (1948).
[8] S. G. Ellis, J. App. Phys. **18**, 846 (1947).

TABLE I.

| Element deposited | Ease of removal |
|---|---|
| Beryllium | − |
| Aluminum | − |
| Chromium | − |
| Manganese | − |
| Nickel | ± |
| Copper | ± |
| Rhodium | + |
| Palladium | + |
| Silver | ± |
| Indium | ± |
| Tungsten | − |
| Platinum | + |
| Gold | + |
| Lead | − |
| Uranium | − |

+, Readily; ±, occasionally; −, non-removable.

total film that it could not be stripped. When deposited *after* the gold, they had no effect upon granulation.

An attempt was made to avoid the use of collodion or Formvar in the stripping process, and to use, instead, a thick film (100–200A) of SiO for the supporting membrane. This type of replica was prepared, but no improvement in gold granulation was found.

b. Attempts to stabilize the gold film subsequent to stripping were based upon the hypothesis that the inevitable distortion of the collodion upon electron impact might produce an incipient breaking of the gold film which would lead to agglomeration. Films of SiO were deposited upon either one or both sides of the collodion film, after the gold film replica had been stripped, and although it was found that the *collodion* film was stabilized thereby, the gold aggregated into droplets as before.

In conclusion to this set of trials, we can say that the only feasible means of reducing gold granulation appears to be the well-known procedure of decreasing the intensity of the electron beam.

### C. Experiments with Pre-Shadowing Agents Other Than Gold

#### *a. Factors Affecting the Adherence of Films to Glass Substrates*

A material satisfactory for use in forming pre-shadowed replicas from a glass surface must be of high electron scattering-power, of non-granulating character, and should be readily removable from the glass by the ordinary collodion stripping technique. In addition, it is convenient if the material is readily evaporated from a tungsten filament in an ordinary shadow-casting equipment. The first requirement restricts us to the use of elements, or compounds, of average atomic number around 60 or greater, while the second requirement apparently restricts us to the use of materials of high melting point, above 1500°C. The nature of the restrictions consequent upon the third requirement (removability of the film) is not very well understood, and most of our efforts have been spent in elucidating this point further.

The relative adherence of evaporated films of metal to glass substrates has been for many years a problem of great practical importance and of extreme empiricism. One of us[9] had found in 1934 that a film of a relatively non-adherent metal, such as gold, could be rendered strongly adherent to glass by the pre-application of a thin film of chromium or beryllium, but the cause of this effect is still obscure. We have made some systematic observations of the tenacity of metal films to glass, and have the following general conclusions to offer.

Gold will normally adhere very weakly to glass, and a gentle pull applied to a film of collodion, dried in contact upon it, will strip it from the glass. The character of the glass surface prior to the deposition of the gold is important, however, and we have found that the surface can be prepared in ways such as to cause the gold to adhere quite firmly. One way is to clean the glass strongly with a chromic-acid cleaning solution; this probably etches the glass surface and creates crevices in which the gold is mechanically anchored. Another way is to cause the surface of the glass to be strongly hydrophobic at the time the gold is deposited. This can result from the presence of a thin film of oil on the surface (applied by rubbing, or even by using an inadequately trapped diffusion pump operating with Silicone oil), or by ionic bombardment within the vacuum chamber. Whatever the means of destroying the natural hydrophilic surface character of clean glass, we have made

[9] R. C. Williams, Phys. Rev. 46, 146 (1934).

the general observations that gold (and other ordinarily non-adhering elements) will adhere to glass if its surface is not readily wet with water.

In speculating upon the causes of this effect, it should be remembered that no metal film can be stripped from glass with the aid of collodion unless the combination has been thoroughly moistened, usually with condensed breath moisture. Possibly it is necessary that water molecules penetrate the collodion, and enter as a film between the metal and the glass in order that the former can be stripped, and if the glass surface is hydrophobic the entry of water will not occur. This hypothesis is supported by the observation that a gold film, coated with film of SiO (which is relatively impervious to water in a thickness of 100A), can be stripped only with difficulty.

*b.*

We have experimented with several materials other than gold in an attempt to discover what characteristics of the material determine its relative adherence to glass, when deposited as a film about 20A thick. Most of our work has been with elements, rather than compounds, and we will report results with these first.

Thin films of approximately 20A thickness were deposited upon clean* microscope-slide glass, and their relative adherence tested by their resistance to removal by means of stripping with a film of collodion. The results following in Table I were obtained.

These data reveal only one reasonably consistent correlation between the character of the element and the relative tenacity; those elements which readily oxidize on exposure to air adhere strongly, while those which do not oxidize are readily removed from the glass. There appears to be no correlation with atomic number, hardness, density, melting point, or crystal structure of the elements. If the generalization is correct, it leads one to anticipate that films of vacuously deposited oxides would be strongly adherent to the glass. We have tested this prediction with the oxides of aluminum, silicon, tungsten, lead and uranium, and have found all of them tightly adherent.

TABLE II.

| Element | Melting point | Atomic number | Specific gravity | Evaporation characteristic | Granulation |
|---|---|---|---|---|---|
| Pd | 1555 | 46 | 12.0 | Simple | Very slight |
| Pt | 1755 | 78 | 21.4 | Difficult | None apparent |
| Rh | 1955 | 45 | 12.5 | Very difficult | None apparent |

The reasons for this selective behavior can be only surmised at this time. We believe that the effect of water or water vapor is an important and ever-present one, since collodion and Formvar films alone cannot readily be stripped from clean glass without having been moistened with condensed water vapor. It is conceivable that a monofilm of water is absorbed between the metal or metal-oxide film and the glass (mostly $SiO_2$), and that in this case hydration occurs with the formation of a hydrogen bond between the oxide layers.

*c. Results with Removable Films*

Our work indicates three conceivable solutions of the problem of producing films for use in pre-shadowed replicas which will meet the requirements listed under "*a*" of this section. The first is to find a smooth substrate from which even oxide films can be removed. (This would allow one to use uranium, for example, as a shadowing agent.) The second is to use a removable combination of oxidizing and non-oxidizing films. The third is to prepare only films which do not oxidize for use as the shadowing agents. We have experimented with all three solutions.

1. There appear to be very few natural or artificial surfaces which are satisfactory for use as pre-shadowing substrates, and are also sufficiently smooth for our purposes. Natural crystals, when cleaved, show relatively gross imperfections of structure, and when polished exhibit the polish marks of the abrasive. Materials which are even slightly soluble in water are generally unsuited for use with the aqueous solvents employed in biological investigations. Polished metals, without exception, exhibit polish marks, and also show their polycrystalline nature

* The glass microscope slides were cleaned by first scrubbing them vigorously with Dreft, followed by thorough rinsing with distilled water. The wet, rinsed slides were then held in steam issuing from a water still. This heated them to the point that they dried almost instantly upon removal from the steam.

when the debris formed by polishing is removed. Mica might appear to be a possibility, but we have found that its behavior with respect to deposited oxide films is like that of glass. In summary, we have found no surface, except glass, that is sufficiently smooth and insoluble to be used as a substate for forming pre-shadowed replicas.

2. It is conceivable that one can effect a combination of a film which is readily removable with one which is not, and utilize the removable feature of the former with the non-granulating feature of the latter. We have tried this possibility, using gold as the removable component, and the oxides of several elements as the non-granulating components. Without exception it is found that when the gold is present in sufficient quantity to allow the film to be removed the resulting combination film exhibits granulation.

3. The third possible solution has at least three variations, all of which we have tried. The first variation is to deposit a film of an oxidizable material, such as uranium, and to prevent its subsequent oxidation by protecting it, or by combining it with an element other than oxygen.

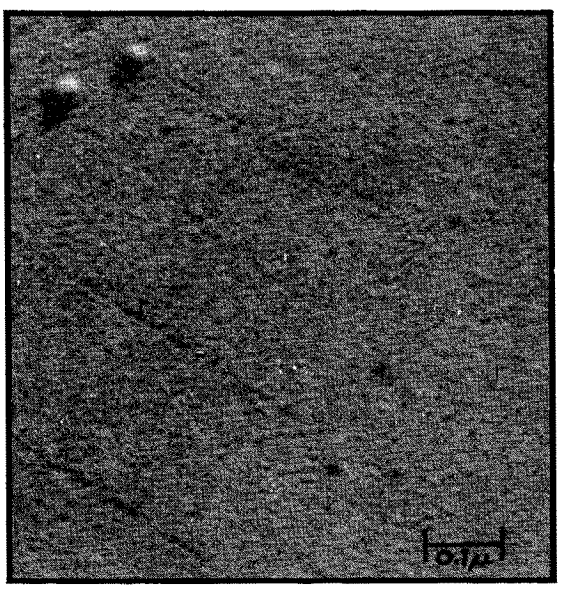

FIG. 3. A pre-shadowed replica of a glass microscope slide upon which palladium-platinum was deposited in a measured thickness of 6A at a shadowing angle of 5 to 1. Area shown in micrograph has more fine scratches than normally encountered. The depth of the scratch at the bottom of the picture is calculated to be about 20A. Magnification the same as in Fig. 1.

Protection was attempted in our work by admitting dry hydrogen to the vacuum chamber, subsequent to the uranium shadow-casting, and also by coating the uranium film, while still in vacuum, with a film of SiO. In both cases the uranium film oxidized, as demonstrated by its tenacity to glass, and by its electron diffraction pattern. The method of substitution for the prevention of the oxide film proved to be more promising. In this case the uranium film was either exposed to $H_2S$ while still in the vacuum chamber, or was quickly dipped into a solution of $H_2S$ in Formvar upon removal from the vacuum chamber, in an attempt to form a uranium sulfide film. The uranium film, so treated, proved to be frequently removable, although the results were not consistent.

The second variation is to deposit films of compounds which are not oxides, but which are stable upon vacuum evaporation, and do not subsequently change to oxides. Inasmuch as we are interested only in the high melting-point compounds of substances of high electron scattering-power, we have tried only the use of uranium sulfide. It evaporates with some difficulty, but it does not alloy with the tungsten filament. However, we have found its use somewhat uncertain, in that it can only occasionally be stripped from the glass substrate by the collodion replica technique.

The third variation is the obvious one using only elements which do not oxidize, and which are also satisfactory in other respects. We have made films of palladium, platinum and rhodium whose pertinent characteristics are indicated in Table II.

Platinum appears to be the most likely element, in respect to its high atomic number and density, and its freedom from granulation. The only limitations to its use are the very high temperature at which it evaporates in a vacuum, and the vigorous alloying action of molten platinum upon tungsten. We have attempted to reduce these difficulties by the use of a small admixture of palladium (about 20 percent by weight) with the platinum. The palladium melts relatively easily and quickly, and helps to melt the platinum by creating good thermal contact between filament and platinum. The net result is that the Pd-Pt alloy evaporates without the

usual spattering associated with the evaporation of pure platinum. It appears that a film of Pd-Pt is generally satisfactory for use with pre-shadowed replicas, and it is of interest to investigate the proper thickness of film to use. Shadowing should be done with a film of thickness sufficient only to establish adequate contrast in the shadows, since a thicker film produces a non-informative black-or-white micrograph and also results in distortion of the shapes of the shadowed particles. Naturally, what constitutes a shadow of minimum contrast is a matter of taste, and a function of the degree to which one is willing to enhance contrast by photographic means. It should be pointed out, however, that the production of vivid shadows by the use of a thick metal film is not consistent with undistorted micrography of particles of a size approaching the ultimate size resolvable by the electron microscope.

Rough calculation shows that a film of platinum should be as effective an electron scattering agent, per unit thickness, as is a film of uranium, since the latter always grows to the oxide with a consequent reduction of average atomic number and density. The effect of the palladium is to reduce the electron scattering power per unit thickness of the platinum, but in the relative amount used by us the effect of the dilution factor is less than 10 percent. We have measured the thickness of a Pd-Pt film just adequate for shadowing. Two microscope slides were placed in the vacuum chamber, one of which was located 15 cm from the filament, and whose surface made a $\tan^{-1}\frac{1}{5}$ with the surface and filament (commonly called a "5 to 1 angle"). The other slide was placed 10 cm from the filament, with a normal to its surface intersecting the filament. Thus the latter surface received about 10 times as thick a film as the former. Replicas were made of the inclined surface, and a micrograph of one is shown in Fig. 3. A collodion stripping was also made of the closer surface, covered by the thicker film, and the slide reinserted in the vacuum chamber. It was then shadowed at a 5 to 1 angle, and a collodion replica obtained. It was found that where there was an occasional patch of the thick Pd-Pt film which resisted the first stripping, it was coated upon re-shadowing the glass, and was then removed by the second stripping. A micrograph of such a patch, shows it to be shadowed, and by measuring the length of the shadow, one obtains the thickness of the patch. This thickness, divided by 10, gives the thickness of the film responsible for the shadows in Fig. 3. It is found that the thickness, so determined, is about 75 percent of that calculated from the geometry of the shadowing arrangement and the known quantity of Pd-Pt used. We conclude, that a calculated film thickness of 5A of Pd-Pt will yield perceptible shadows of small objects.

It is worth while to call attention again, as demonstrated by Williams and Wyckoff,[10] to the extreme smoothness of a glass surface. Our trials indicate that there are no perceptible differences in fine-scale smoothness between glass surfaces which are commercially rouge-polished or fire-polished, as long as the surfaces are not old enough to be atmospherically etched. The rouge-polished surfaces will show infrequent gross scratches, of course, of the order of 500A in depth. It appears that there are rarely sharp discontinuities in elevation as large as 5–10A on the surface of any freshly polished glass.


### ACKNOWLEDGMENT

This work has been done as a preparatory phase of an electron micrographic study of the physical properties of large biological molecules. It was supported in large part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.


### APPENDIX

#### 1. The Shadow-Casting Process

Some descriptions of the shadow-casting process have been published[11] but no comprehensive and detailed discussion of the technique seems to exist.

a. The proper *substances* to use for shadowing have been discussed in this paper and elsewhere,[12] but in summary this can be said: For general purposes, where detail of structure of roughly 200A or larger is of interest, chromium or manganese are the most convenient and reliable elements to use. For the elucidation of contours of relatively large objects, such as bacteria and fibers, the shadowing films

[10] R. C. Williams and R. W. G. Wyckoff, Science **101**, 594 (1945).
[11] R. C. Williams and R. W. G. Wyckoff, J. App. Phys. **15**, 712 (1944), J. App. Phys. **17**, 23 (1946).
[12] W. T. Dempster and R. C. Williams, Anatomical Record **96**, 27 (1946).

should be applied at an angle of about 4 to 1, and in a computed thickness of about 60A. If cast upon a collodion substrate at angles more oblique than about 6 to 1, they will build up into a distinctly rough-appearing layer.

For fine detail of structures which are mounted on film substrates, or for the visual rendition of very small particles similarly mounted, the best materials appear to be uranium or uranium oxide. They will cast perceptible shadows in a computed thickness of about 4A, and intense shadows in a thickness of 8A. The most generally useful shadow-casting angle here is 5 to 1.

For pre-shadowed replicas, palladium-platinum or platinum alone appear to be most useful, in thicknesses about the same as for uranium. Gold is very convenient, and is usually satisfactory if not made thicker than about 8A and used in an instrument with a non-biased-beam electron gun. Granulation is made evident by the appearance on the micrographs of small globules which cast no shadows.

b. The calculated *thickness* of films is reliable, if an ordinary conical filament is used for evaporation and if an efficiency factor of about $\frac{3}{4}$ is allowed. The thickness is calculated to be that which exists on a plane surface making an angle $\alpha$ with the direction line to the filament. The formula for calculation is:

$$t=\frac{3}{4}\frac{M\cdot\tan\alpha\cdot 10^8}{4\pi r^2\cdot d},$$

where: $t$=thickness in Angstrom units, $M$=mass of material in grams, $\alpha$=angle of shadow-cast (defined above), $r$=distance in cm from filament to specimen, $d$=density of material in g/cc.

A convenient value for $r$ is 15 cm, and for $\alpha$ is $\tan^{-1}\frac{1}{6}$. A thickness of 4A of uranium is then calculated to require about 12 mg of the element. It should be noted, of course, that any element of surface exposed at perpendicular incidence to the filament will receive, in this case, approximately 5 times as thick a deposit. This heavy deposit will distort the shapes of very small objects, by causing them to be somewhat elongated ellipsoids (if they are originally spheres) in the direction of the shadow-casting filament, and to become invisible beyond the "sunset edge" on the side away from the filament. A good estimate of the particle diameters, however, can be obtained by measurements perpendicular to the direction of shadowing.

c. The *evaporation* of chromium, manganese, gold, and uranium oxide is most easily performed from a 15–20 mil tungsten filament wound in the form of a conical helix, or basket, of three or four turns, and heated with about 20 amperes of current. The forming of the filament is most easily done by winding on a No. 4–6 woodscrew, with the tungsten and screw as hot as the hand will tolerate. The specimen can be mounted to receive the evaporating atoms either from the top or from the side of the conical filament.

Molten uranium and platinum wet and dissolve the filament, and a larger size wire, 30 mil, is desirable to prevent premature filament breakage. About 45 amperes of current are required to heat the filament adequately. A convenient filament form for the evaporation of these two elements is a sharp hairpin pointed directly toward the specimen, and with its apex bent up through 90°. The molten droplets will at first oscillate back and forth along the arms of the hairpin, but will finally come to rest and evaporate from the apex.

It is best to place the calculated amount of material in the filament and to evaporate it completely. The evaporating material should subtend as small a solid angle as possible at the specimen, in order to retain the sharpest possible shadow edge.

A vacuum satisfactory for vacuum aluminizing (about $10^{-4}$ mm mercury as measured on a Pirani gauge) is adequate for shadowcasting. The best criterion of excellence of vacuum, however, is the sharpness of the shadows, since too high a residual pressure will result in indistinct shadows. As indicated previously in this paper, Silicone oil should not be used in the diffusion pump, if pre-shadowed replicas are to be made, but Octoil is satisfactory.

d. The production of replicas of specimen mounted on glass: The technique for the stripping of collodion or Formvar replicas, with the aid of Scotch Tape, was described some years ago.[13] We have found this method very satisfactory, and can contribute little in addition. Collodion dissolved in amyl acetate, or Formvar dissolved in ethylene dichloride, can be applied either as a single drop on the pre-shadowed specimen held almost vertically, or the whole pre-shadowed glass slide can be immersed in the liquid and then allowed to dry while vertical. In either case, a satisfactory thickness of substrate film seems to be such that the film, while on the glass surface, appears to have a faintly purple hue.

The specimen screens for the final mounting of the replica are located on a strip of Scotch Tape, with a small piece of paper between the tape and the middle of the screen to avoid contact between the center of the screen and the tape. With wire-mesh screens the concave side should be toward the tape. The tape with specimen screen is held ready, and the moisture from one's breath is caused to condense on the collodion-coated glass slide. The condensate should be quite heavy, and there appears to be no danger of overdoing this. The tape with specimen is then quickly pressed down on the moisture-coated specimen, and pulled off fairly rapidly but gently. The successful removal of the pre-shadowing metal film can be detected prior to insertion of the specimen in the microscope by noting whether or not the stripped area of the glass slide appears more transparent than adjacent areas, since a film of gold or palladium-platinum only 5A thick has detectable optical opacity.

The specimen grid is removed from the Scotch Tape by gently running a sharp object, like a needle, around its periphery, and then removing the grid with flat-nosed tweezers. We have found that, for some obscure reason, the stripping of replicas from glass is most successful when done as soon as feasible after the shadow-casting and the application of the collodion film. No systematic difference in the technique of replica removal appears to exist between collodion and Formvar.

[13] V. J. Schaefer, Phys. Rev. 62, 495 (1942).